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Review

Virus-induced gene silencing: A versatile tool for discovery of gene functions in plants

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ABSTRACT

Virus-induced gene silencing (VIGS) is a technology that exploits an antiviral defense mechanism in plants as a tool for plant reverse genetics. VIGS circumvents the need for plant transformation, is methodologically simple and yields rapid results. Various VIGS vectors have been developed and have helped to unravel the functions of genes involved in processes such as disease resistance, abiotic stress, cellular signaling and secondary metabolite biosynthesis.

1. Introduction

Virus-induced gene silencing (VIGS) is rapidly emerging as a method of choice for rapid silencing of plant genes in order to decipher their function. The popularity of VIGS can be attributed to four reasons. First, the methodology is simple often involving agroinfiltration or biolistic inoculation of plants. Second, the results are obtained rapidly typically within two-three weeks of inoculation. Third, the technology bypasses transformation steps and hence is applicable to number of plant species recalcitrant to transformation. Fourth, the method has the potential to silence multi-copy genes.

VIGS is based upon the phenomenon of RNA-interference (RNAi), which refers to interference in gene expression, mediated by small RNA in a sequence specific manner. Manifestations of this pathway are variously termed as post-transcriptional gene silencing (PTGS) in plants, quelling in fungi and RNAi in animals. One important function of this pathway in plants is in defense against viruses, in which, viral RNA acts as a trigger to induce RNA-mediated gene silencing which, in turn, is directed against viral genes. In VIGS, this viral RNA-induced defense mechanism against viruses has been exploited as a tool for reverse genetics and analysis of gene functions in plants, known as VIGS. As compared to other PTGS-based methods requiring genetic transformation steps, a "functional knock-down" for a plant gene can be created using VIGS within a matter of weeks without having to transform plants.

Several RNA and DNA viruses have been modified to develop VIGS vectors. The gene to be silenced is cloned in an infectious derivative of a viral DNA (DNA virus-based vectors) or cDNA (RNA virus-based vectors) derived from viral RNA. The VIG vectors are introduced into plants by mechanical inoculation of *in vitro* transcripts, *Agrobacterium*-mediated agroinfiltration or, for DNA-based vectors by biolistic delivery methods. During the course of viral infection, either double-stranded RNA or RNA with high degree of secondary structure is often produced, both of which are efficient initiators of RNAi directed against the infecting viral RNA [42,77,85]. Although direct evidence is still lacking, a similar phenomenon is thought to operate in VIGS, where RNAi is directed both against the viral genome in the VIGS vector and host transcript bearing sequence similarity to the inserted DNA fragment. This results in the degradation of the target transcript leading to silencing of the corresponding target gene. In addition to sequence homology other factors are necessary to initiate efficient RNAi in VIGS. It was reported that a DNA fragment with a minimum of 23 nucleotides bearing 100% identity to a targeted transgene is sufficient for silencing to occur [105]. However, other reports suggest that longer sequences are required [32], indicating that unknown factors modulate this effect. Other factors that play an important role in gene silencing in VIGS are the orientation of the insert; inverted repeats being more efficient than in antisense orientation, which, in turn is more efficient than the same in sense orientation [46,64]. The silencing signal is believed to spread independent of the VIGS vector to other parts of the plant, leading to systemic spread of the silencing effect (Fig. 1).

Besides being simple and rapid, VIGS is particularly useful in assessing gene functions in species recalcitrant to transformation

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and genes that cause embryo lethality in knock-outs. Another advantage of the VIGS technique is that it can be designed to silence multiple members of a gene family, thereby circumventing the problem of functional redundancy of genes.

2. VIGS: the fast-forward tool for reverse genetics

The term VIGS was first used to describe the phenomenon of recovery from virus infection in plants [108]. Today, the term has become synonymous with a technique in which a recombinant viral vector is used to knock-down expression of endogenous genes [94,10]. VIGS, together with biochemical and genetic studies, has been extensively used to decipher functions of a large number of genes belonging to growth and development, defense response pathway and abiotic stress response in plants in recent years. Most importantly, it has been possible to study gene functions in plant species for which transformation protocols are not yet standardized.

The first VIGS vector was developed using an RNA virus, *Tobacco mosaic virus* (TMV). Phytoene desaturase (PDS) is an enzyme

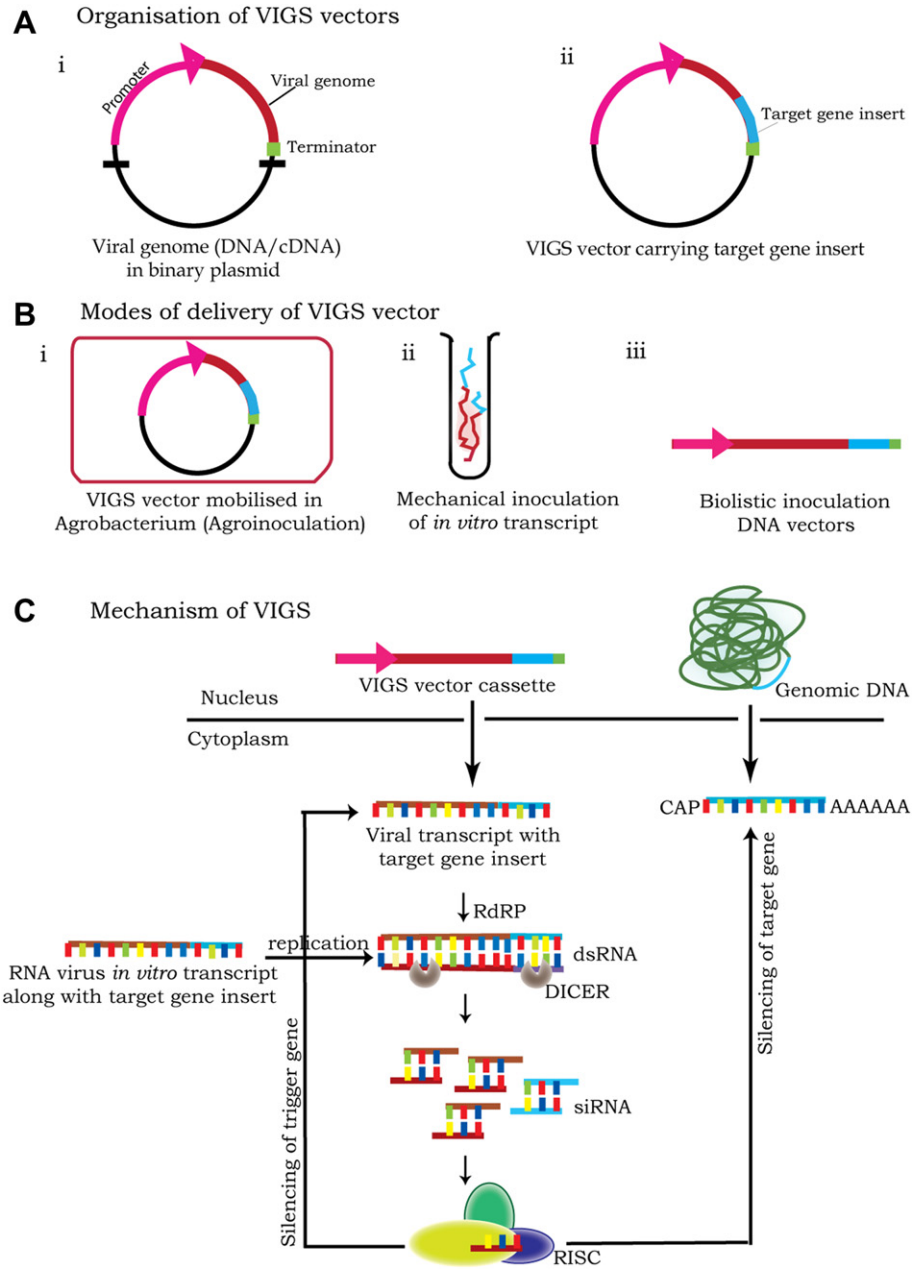

Fig. 1. Structure, mode of inoculation and mechanism of VIGS. A. Schematic representation of DNA-based VIGS vector, i) Genome of DNA virus or cDNA of RNA virus driven by constitutive promoter, ii) VIGS vector carrying target insert, B. Modes of inoculation of VIGS vectors, i) VIGS vector carrying target insert transformed into *Agrobacterium* can be introduced into plants by agroinoculation procedures, ii) *In vitro* transcripts of RNA virus carrying target insert can be introduced into plants by mechanical transmission, iii) Promoter-insert-terminator constructs can be introduced into the plants by biolistic bombardment, C. Mechanism of virus-induced gene silencing. Replication intermediates of RNA viruses and viral transcripts derived from DNA-based vectors amplified to dsRNA by host RdRPs are efficient triggers of RNA silencing. DsRNA is processed to give rise to 21–24nt siRNAs, which act as the guide molecules. A single strand of siRNA (guide strand) is loaded into the multiprotein effector complex (RISC). Using siRNA as guide, components of RISC bring into effect homology-dependent degradation of target transcripts. RdRP: RNA dependent RNA polymerase, dsRNA: double-stranded RNA, siRNA: short interfering RNA, RISC: RNA-induced silencing complex.

participating in the biosynthesis of carotenoid, compounds that protect chlorophyll from photo-bleaching. Transcripts of recombinant TMV carrying a sequence encoding *pds* were produced *in vitro* and inoculated to *Nicotiana benthamiana* plants, leading to the successful silencing of *pds*, the silenced plants displaying photo-bleaching of the leaves [63]. Since then, many viruses have been modified to develop VIGS vectors (Table 1). The fact that certain VIGS vectors can be used to silence genes in non-host plants, provided the system supports the replication of the viral vector, is advantageous, because in that way a single VIGS vector can be used to silence genes in several plant species (Table 1). Apart from *N. benthamiana*, a plant highly susceptible to viral infections and hence easily amenable to VIGS, the technique has been extended to plants such as tomato, petunia, Arabidopsis and barley, which have been covered in several excellent reviews [14,73,91]. This review deals with recent modifications in the VIGS technique, the use of new VIGS vectors on hitherto untested plant species and its use in unraveling the function of novel genes, mostly related to the defense response pathway in plants.

3. Development of new viruses as VIGS vectors

Since its first demonstration about seven years ago, VIGS has been used extensively in solanaceous species such as petunia, tobacco, tomato and in the model plant Arabidopsis, using vectors based on *Potato virus X* (PVX), TMV and *Tobacco rattle virus* (TRV), all having RNA genomes. Subsequently, VIGS has been extended to several new plant species using new viruses adapted to them (Table 1). For example, to extend VIGS to cassava (*Manihot esculenta*), an important tuber crop, a VIGS vector was developed from the DNA virus *African cassava mosaic virus* (ACMV). Biolistic inoculation of cassava plants with the ACMV-based vector carrying the gene encoding a sub-unit for the chlorophyll synthesis enzyme magnesium chelatase (su), resulted in development of yellow-white spots in the leaves characteristic of *su* silencing [34]. Subsequently, ACMV-VIGS was used to silence the enzyme required for the biosynthesis of a harmful cyanogenic glycoside linamarin in cassava, resulting in its significant reduction in the leaves.

For extending the use of VIGS to pea, a derivative of the RNA virus *Pea early browning virus* (PEBV) was used [24]. Using PEBV-VIGS, several genes such as, *pds*, a gene involved in regulating compound leaf architecture, *uni* (a homolog of the *flo* and *lfy* genes from *Antirrhinum majus* and Arabidopsis respectively) and a gene involved in maintaining cell wall architecture, *korrigan1* (encoding endo-1,4-β-D-glucanase) in addition to the marker gene Green fluorescent protein (*gfp*) were silenced. Pea plants silenced for *pds* showed characteristic photo-bleaching spots within 9–10 days post-inoculation (dpi). At 42 dpi, more than 50% of the *uni*-silenced plants exhibited distorted leaf development, in some cases the tendrils developing into leaflets, the phenotype resembling those observed on genetic *uni* mutants. Similarly, *korrigan1*-silenced plants displayed stunting. Silencing of more than one gene at a time was successful but the phenotype was milder than when silenced individually.

Another legume-infecting RNA virus, *Bean pod mottle virus* was used for VIGS in soybean and has been tested by silencing *pds* [116]. Extracts from the *pds*-silenced leaves were used for further inoculation. A *Cucumber mosaic virus*-derived VIGS vector has also been developed for silencing of genes in soybean. The vector has been used to silence chalcone synthase (*chs*) and sf3′h1, which is predicted to encode flavonoid 3′-hydroxylase (F3′H), in soybean [79]. Plants silenced for *chs* displayed loss of pigmentation in the seed coat. Similarly, silencing of sf3'h1 was associated with decreased levels of flavonol in the silenced plants.

Several monocotyledons plants, including rice, maize and barley are the natural hosts for the RNA containing *Brome mosaic virus* (BMV). A BMV-derived VIGS vector was used to silence *pds* counterpart in barley [29]. In addition, BMV-VIGS was used to silence actin and rubisco activase genes in rice. Extension of VIGS to rice is expected to play an important role in the functional characterization of this model cereal plant, whose genome is fully sequenced [51].

A number of viruses are associated with symptom-modulating satellite molecules, which, by nature, are totally dependent upon the virus for replication. An asymptomatic satellite DNA associated with the DNA virus *Tomato yellow leaf curl China virus* has been modified as a VIGS vector for use in *N. benthamiana* [104]. In the presence of the helper virus, the modified satellite, carrying *gfp*, *su* or *pds* caused efficient silencing of the corresponding genes in inoculated plants. In addition, silencing of the gene encoding the essential replication protein PCNA indicated that silencing was active even in the meristematic tissue, a region, which is generally believed to be free from virus infection. This strongly indicated that the spread of the silencing signal can take place independent of the VIGS vector and opened the possibility of silencing genes related to the development and differentiation of plant meristems. Another satellite, the DNA1 component of *Tobacco curly shoot virus* has been recently modified for use as a VIGS vector and has been used successfully in *N. benthamiana* [49]. *Turnip yellow mosaic virus*-based VIGS vector has been used to silence *pds* and the meristem-expressed gene, *lfy* in the model plant Arabidopsis [88].

Recently, a VIGS vector derived from the tree-infecting *Poplar mosaic virus* (PopMV) that has been tested on *N. benthamiana* for silencing a transgenically-expressed *gfp* gene [80]. Another tree-infecting virus to be developed into a VIGS vector is based on *Apple latent spherical virus* (ALSV). Both PopMV and ALSV are RNA viruses. The ALSV-VIGS vector has been used to silence endogenous genes among a broad range of plants including tobacco, tomato, Arabidopsis, cucurbits and legumes [111,50]. The ALSV-based vector has also been proved useful to study gene silencing in soybean seeds and in emergence stage of soybean plants [112]. The vector led to significant reduction in the levels of *pds* in plants inoculated with ALSV vector at the two-cotyledon emergence stage and also in the seeds borne by *pds*-silenced plants. Isoflavone synthase (IFS), a key enzyme in the formation of isoflavones and encoded by two genes in soybean (*IFS-1* and *IFS-2*), has been silenced in soybean. Infection of soybean seeds with ALSV-based vector carrying a fragment *ifs* gene led to a decrease in the levels of transcript of isoflavone in the cotyledons of infected seeds. However, the use of PopMV- and ALSV-VIGS vectors in the functional genomics of tree species is yet to be demonstrated. Interestingly, *Grapevine virus A* (GVA) which causes Rugose wood disease of grapevine has been used as a VIGS vector [78] to silence *pds* in *N. benthamiana* and in micropropagated grapevine plants plantlets.

4. Improvement of existing VIGS vectors to perform specialized tasks

Apart from developing VIGS vectors based on new viruses, existing vectors (mostly TRV-based), have been modified to increase their silencing efficiency and to perform specialized silencing tasks. For example, spray technique was used with the TRV-based vector for tomato [68]. This was found to be more efficient in case of tomato plants compared to the usual infiltration methods. The method was used to silence *pds* and the *ctr1* (Constitutive triple response-1) genes.

To allow high-throughput insertion of Expressed sequence tags (ESTs), such as those from tomato, a restriction- and ligation-free cloning method, using the GATEWAY recombination system, was incorporated into TRV vector allowing the functional screening of

Table 1
Viruses used to develop VIGS vectors, nature of their genomes and the genes silenced.

Virus/satellite	Genus	Nature of genome	Important natural hosts	Silenced hosts	Genes silenced	References
African cassava mosaic virus	Begomovirus	ssDNA, bipartite	*Manihot esculenta*	*N. benthamiana*, *M. esculenta*	*pds*, *su*, *cyp79d2*	[34]
Apple latent spherical virus	Cheravirus	Positive-strand RNA, bipartite	Apple	*N. tabacum*, *N. occidentalis*, *N. benthamiana*, *N. glutinosa*, *Solanum lycopersicon*, *A. thaliana* Cucurbit species, several legume species	*pds*, *su*, *pcna*	[50]
Barley stripe mosaic virus	Hordeivirus	Positive-strand RNA, tripartite	Barley, wheat, oat, maize, spinach	*Hordeum vulgare*	*pds*	[48]
Bean pod mottle virus	Cucumovirus	Positive-strand RNA, bipartite	*Phaseolus vulgaris*, *Glycine max*	*G. max*	*pds*	[116]
Brome mosaic virus	Bromovirus	Positive-strand RNA, tripartite	Barley	*Hordeum vulgare*, *Oryza sativa* and *Zea mays*	*pds*, *actin 1*, *rubisco activase*	[29]
Cabbage leaf curl virus	Begomovirus	ssDNA, bipartite	Cabbage, broccoli, cauliflower	*A. thaliana*	*gfp*, *CH42*, *pds*	[106]
Cucumber mosaic virus	Cucumovirus	Positive-strand RNA, tripartite	Cucurbits, *S. lycopersicon*, *Spinacia oleracea*	*G. max*	*chs*, *sf3′h1*	[79]
Pea early browning virus	Tobravirus	Positive-strand RNA, bipartite	*Pisum sativum*, *Phaseolus vulgaris*	*P. sativum*	*pds*, *uni*, *kor*	[24]
Poplar mosaic virus	Carlavirus	Positive-strand RNA, monopartite	Poplar	*N. benthamiana*	*gfp*	[80]
Potato virus X	Potexvirus	Positive-strand RNA, monopartite	*Solanum tuberosum*, *Brassica campestris* ssp. *rapa*	*N. benthamiana*, *A. thaliana*	*gus*, *pds*, *DWARF*, *SSU*, *NFL*, *LFY*	[94]
Satellite tobacco mosaic virus	RNA satellite virus	RNA, satellite	*Nicotiana glauca*	*N. tabacum*	Several genes	[41]
Tobacco curly shoot virus DNA1 component	DNA satellite-like virus	DNA, satellite	*N. tabacum*	*N. tabacum*, *Solanum lycopersicon*, *Petunia hybrida*, *N benthamiana*	*gfp*, *su*, *chs*, *pcna*	[49]
Tobacco mosaic virus	Tobamovirus	Positive-strand RNA, monopartite	*N. tabacum*	*N. benthamiana*, *N. tabacum*	*pds*, *psy*	[63]
Tobacco rattle virus	Tobravirus	Positive-strand RNA, bipartite	Wide host range	*N. benthamiana*, *A. thaliana*, *S. lycopersicon*	*pds*, *rbcS*, *FLO/LFY (NFL)*	[70,89]
Tomato golden mosaic virus	Begomovirus	ssDNA, bipartite	*S. lycopersicon*	*N. benthamiana*	*su*, *luc*	[87]
Tomato yellow leaf curl China virus– associated β DNA satellite	Begomovirus	ssDNA, satellite	*S. lycopersicon*	*N. benthamiana S. lycopersicon*, *N. glutinosa*, *N. tabacum*	*pcna*, *pds*, *su*, *gfp*	[104]
Turnip yellow mosaic virus	Tymovirus	Positive-strand RNA, monopartite	Brassicaceae	*A. thaliana*	*pds*, *lfy*	[88]

a large number of gene fragments. Using this method it was demonstrated that silencing of the small sub-unit of the ribulose bisphosphate carboxylase (*tRbcS*) and an endogenous gene corresponding to tomato EST *cLED3L14*, (showing high homology to potato protein kinase, StCPK1), resulted in the production of pale yellow leaves in the case of *rbcS* silencing. However, the cLED3L14-silenced plants showed no visible phenotype despite approximately 80% reduction in the transcript level [68]. The use of TRV-based vector has been optimized for efficient silencing of *pds*, *chlorata 42* (involved in chlorophyll biosynthesis) and *cullin* (ubiquitin ligase) resulting in yellowing and stunting in Arabidopsis [15]. Another modified ligation-independent cloning strategy (LIC) [8,28] has been described (TRV-LIC) [30]. Using this strategy several ESTs of tomato encoding kinases, transcription factors and phosphatases were silenced in *N. benthamiana*.

As a specialized adaptation to silence fruit specific genes, TRV-based vector was used to efficiently silence genes directly in the tomato fruit [35]. Syringe-infiltration into the carpodium of the attached fruit was found to be more effective over syringe-infiltration into the stem, surface of the attached fruit or vaccum-infiltration into a detached tomato fruit. This method was used to silence several genes of the ethylene-signaling pathway (*leacs2*, *lectr1*, *leeils* and *leein2*). Subsequently, silencing of *pds* directly in various developmental stages of tomato fruit using TRV-vector was demonstrated [82].

A modified TRV-vector has been used to efficiently silence root-specific genes [107]. in which the inclusion of the 2b, the viral helper protein, in the TRV vector (TRV-2b) greatly enhanced the ability of the TRV-VIGS vector to invade the roots of *N. benthamiana* and Arabidopsis. Several root-specific genes including those involved in root development and lateral root meristem function were efficiently silenced by this modified TRV-vector. Using this method it was demonstrated that silencing of *Mi-1* correlates with the nematode susceptibility, hence implicating the gene in determination of nematode resistance.

To extend the use of TRV-VIGS vector to diverse plant species, its use has been optimized for efficient silencing of endogenous genes in several dicots such as Arabidopsis [15,73,110] several solanaceous species [11,23], opium poppy [47], the emerging model plant columbine *Aquilegia vulgaris* [39] and the legumes *Medicago truncatula* and *Lathyrus odarata* [40].

An interesting method has been described for introduction of the TRV-VIGS vector "Agrodrench" into plants in which the Agrobacterium suspension is directly applied into the surrounding soil. The method is more versatile than infiltration as it is a high-throughput system, can be applied to younger plants that have not yet developed fully-opened leaves and is more effective for silencing genes expressed in roots [95]. Simultaneous silencing of several genes was demonstrated by including multiple gene sequences in the same VIGS construct. This has the advantage of silencing of the endogenous gene encoding chalcone synthase (chs), which serves as a marker by giving rise to a visual phenotype upon silencing, and a target gene (such as the senescence-related *aco4*) in the same tissue [18]. This marks a significant technical improvement, as it would be easier to select tissues for analysis of the silencing effects of the target gene, using marker genes as a guide.

Many VIGS vector involve a mechanical inoculation of *in vitro* transcripts, which is somewhat cumbersome. An improvement in the VIGS technology would include development of DNA-based versions, which are easier to handle. For example, the BPMV based vector mentioned earlier has also been modified into a DNA-based version [117]. The first report of the BPMV based vector required *in vitro* transcription and inoculation. Also, infectivity was achieved only with severe strains of BPMV, which led to disease symptoms. In an improved version of the vector, the BPMV genomic elements were placed under the control of CaMV35S promoter and *nos* terminator. Much milder strains of BPMV also resulted in high percentage of infectivity and could be delivered by biolistic bombardment. Several genes, including an actin gene homolog required for the formation of plant cytoskeleton, ribosomal proteins Rps6 and Rps13, defense signaling genes *mpk4A* and *mpk4B* and *sgt1A* and *sgt1B* were successfully silenced using this vector. Simultaneous silencing of *sgt1A* and *sgt1B* was also successfully demonstrated. Silencing of *rps6*, *rps13* and *actin* were associated with stunted root phenotype. The ability to silence genes expressed in roots is particularly useful in leguminous species as roots play pivotal roles in nodule formation and nitrogen fixation.

5. Gene functions revealed by VIGS

A number of gene functions have been revealed by VIGS in the last few years, which have been listed in Supplementary Table I and are described in the following sections.

5.1. Defense response

The basic premise of the Gene-for-gene hypothesis for plant disease resistance [33] consists of recognition of a pathogen-encoded Avr protein by the product of the cognate host R protein, the recognition triggering a local Hypersensitive response-Programmed cell-death (HR-PCD) reaction in most cases. The HR-PCD acts to effectively restrict the pathogen inside dead cells, resulting in resistance. The signal for the presence of pathogen is transmitted to the genes encoding the proteins responsible for HR-PCD by a series of components called the signal transduction pathway. HR proceeds by a burst of reactive oxygen species (ROS), ion fluxes, cell wall strengthening, protein phosphorylation, the accumulation of salicylic acid (SA) and the activation of various defense genes.

One of the first uses of VIGS to unravel genes in defense response pathway was the one triggered by the tobacco *N* gene against TMV. *N* encodes a Toll-interleukin-1receptor/nucleotide binding site/leucine-rich repeat (TIR-NBS-LRR) class protein. VIGS has been used to investigate the role of candidate genes in the N-mediated signaling pathway in tobacco. Transgenic N-containing *N. benthamiana* plants exhibit resistance to TMV by induction of HR lesions to contain the TMV to the infection site similar to the reaction shown by *Nicotiana tabacum* bearing the N gene [70]. *Rar1*, *EDS1* and *NPR1*/*NIM1* are genes known to function in defense response pathway. To investigate their involvement in N-mediated defense pathway they were silenced by VIGS in *N. benthamiana* containing the N gene. Silencing of the genes resulted in TMV infection even in the presence of N gene. Using the same approach it was further established that two MAPK cascade components, *NtMEK1*/*NQK1* and *NTF6*/*NRK1* and the transcription factors WRKY1–WRKY3 and MYB1 play a role in N-mediated resistance [69]. Silencing of vacuolar processing enzyme (*VPE*), a protein that has a proteolytic activity towards a caspase-1 substrate in transgenic N-containing *N. benthamiana* plants, was used to establish its role in TMV-induced HR [44]. Similarly, the role of *NRG1*, which codes for an NB-LRR type R protein in N-mediated resistance, was established in transgenic N-containing *N. benthamiana* [86].

Viruses interact with a number of host proteins for successful replication within the plant cells. One such host protein identified in *N. benthamiana* by yeast-two hybrid assay is the 33 K sub-unit of the oxygen-evolving complex of photosystem II. Silencing of the corresponding gene by VIGS in *N. benthamiana* led to 10 fold increase in the accumulation of TMV indicating a role of the above protein in plant defense pathway [1]. This may reflect a novel

strategy of TMV to suppress basal host defense mechanisms by the above interaction.

In order to be an effective strategy against pathogens, the HR-PCD reaction must be limited to the site of infection and prevented from spreading to uninfected tissue. To identify such genes involved in pathogen-induced HR-PCD, a TRV-VIGS *N. benthamiana* cDNA library screening for altered PCD response to TMV infection showed that BECLIN-1, an *N. benthamiana* ortholog of the yeast and mammalian autophagy gene, functions to restrict HR-PCD [71,66]. Upon TMV infection, the HR-PCD response was unrestricted in the *beclin-1*-silenced plants indicating its role in limiting HR-PCD response to the site of infection.

Resistance against *Cladosporium fulvum*, a fungal pathogen that infects tomato, depends on resistance (*Cf*) genes interacting with pathogen *Avr* genes that encode race-specific elicitors of the pathogen. Transgenic tomato seedlings co-expressing Cf-4 and Avr4 mount an HR at 20 °C, but not at 33 °C. By comparing the expression profile of transgenic tomato plant that contains both the *Cf-4* resistance gene and the matching *Avr4* avirulence gene with that of control plants expressing *Cf-4*, using cDNA-AFLP analysis, 442 Avr4-responsive tomato cDNA fragments (ART) were found to be differentially expressed, of which 192 were selected for VIGS in Cf-4-transgenic *N. benthamiana* [36]. As expected silencing of several ART fragments, resulted in a compromised HR. However, interestingly, silencing of genes encoding a *HSP90*, a nuclear *GTPase*, an L19 ribosomal protein and an NB-LRR type protein (*NRC1*) severely suppressed Avr4-induced HR. Later, the same group also demonstrated that knock-down of the *NRC1* gene also compromises other R protein-induced HR [37]. The R gene, Rx, an NB-LRR protein from potato confers resistance to PVX and interacts with Ran GTPase-Activating Protein2 (RanGAP2). VIGS of *RanGAP2* showed that the interaction with Rx is required for conferring PVX resistance in *N. benthamiana* [103].

The role of a plant papain cysteine protease (Cathepsin B) in disease resistance-triggered HR has already been documented [38]. VIGS-mediated silencing of *NbCathB* resulted in reduction of HR in response to non-host pathogen *Erwina amylovora* in *N. benthamiana*. It also suppressed HR in response to interaction between the R gene of potato, *R3a* and *Phytopthora infestans* Avr3a gene products.

One of the most rapid responses in plants upon recognition of pathogen avirulence factors is oxidative burst, leading to a transient production of large amounts of ROS, including superoxide (O_2^{-2}), hydrogen peroxide (H_2O_2), and the hydroxyl radical. VIGS of two respiratory burst oxidase homologs, *NbrbohA* and *NbrbohB* in *N. benthamiana* have indicated that both are required for H_2O_2 accumulation; their silencing leading to a delayed HR-induced cell death upon challenge by potato pathogen *P. infestans* [115]. Similarly, silencing of two genes from pepper, an extracellular peroxidase and a cytochrome P450 revealed their defense-related roles [21,59].

CCR4-associated factor 1 (CAF1), is known to play an important role in the control of transcription and mRNA decay in yeast and mammals. Silencing of *CaCAF1* in pepper led to growth retardation and increased susceptibility to bacterial spot pathogen *Xanthomonas campestris* pv vesicatoria (*Xcv*) [96]. These results suggest that plant CaCAF1 may play a role in normal growth and development as well as defense against pathogens.

Pectins are components of the plant cell wall and functions as primary barrier against pathogens. VIGS of the gene encoding CaPMEII, a pectin methylesterase inhibitor protein resulted in enhanced susceptibility to *Xcv* and reduced expression of defense-related genes [7]. TRV-VIGS of *CaMNR1* [menthone: (1)-(3S)-neomenthol reductase] gene, a member of the short-chain dehydrogenase/reductase (SDR) superfamily and a novel antimicrobial protein *CaAMP1* in pepper resulted in enhanced susceptibility to *Xcv* as well as *Colletotrichum coccodes* [22,65]. Taken together, these data indicated that *CaPMEII*, *CaMNR1* and *CaAMP1* positively regulate plant defenses responses in pepper. On the other hand VIGS of *CaMRP1*, a novel pepper pathogen-induced gene predicted to encode a membrane-located receptor like protein, conferred enhanced basal resistance to *Xcv* infection indicating that it may act as a negative regulator of basal defense in plants [6].

The role of mitogen-activated protein kinase cascade in innate immunity is well established. MAPK cascades are rapidly activated in plant upon pathogen infection. VIGS has been used to illustrate the role of several members of this cascade, such as NPK1 a MAPKKK [53], NtCDPK2 [92], SIPK, WIPK, NtMEK1, NtMEK2, NtF6, AICK1, LeMPK1, LeMPK2, and LeMPK3 [55,93,101,102] in *N. benthamiana* and in tomato. Their silencing indicated that they participate with R gene-mediated response against a number of pathogens.

Elicitins are highly conserved proteins that are secreted in culture by all tested *P. infestans* and *Pythium* species. *P. infestans* elicitin, such as INF1 causes HR in *N. benthamiana*. VIGS was used to confirm the role of *NbLRK1*, a lectin-like receptor kinase, in INF-mediated HR [58]. Similarly, *LePP2Ac*, encoding a catalytic sub-unit of the heterotrimeric Protein phosphatase 2A (PP2A), was silenced using VIGS in *N. benthamiana*, which resulted in increased resistance to the virulent strain of *pv. tabaci* and accelerated HR. This was true for both *Pseudomonas syringae* and *C. fulvum*. It was thus concluded that LePP2Ac acts as a negative regulator of plant defense response [45].

VIGS was used to obtain a better understanding of the signal transduction components involved in the R gene *rps2*-mediated resistance pathway in the model plant Arabidopsis against *P. syringae* pv. tomato, carrying the avirulence gene *avrrpt2* [17]. Silencing the genes *rar1*, *hsp90* and *ndr1*, known to function in defense response, resulted in development of disease symptoms by *P. syringae*, even in *rps2*-expressing plants, indicating the positive roles played by the silenced genes in the pathway. Similarly, silencing the gene *rin4* resulted in an enhanced resistance response and hence, was concluded to play an inhibitory role in the pathway.

VIGS was used to demonstrate that the cell death in Arabidopsis, carrying the R gene *rps4*, upon infection with *P. syringae* is dependent on the three plant signaling components EDS1, SGT1 and HSP90 [118]. Similarly, the role of HSP90 in plant disease resistance, presumably by its requirement for the proper folding of the protein components of the disease resistance pathway was demonstrated using VIGS [72]. The R gene, *i-2* confers resistance against the fungal pathogen *Fusarium oxysporum* in tomato. It was seen that VIGS-mediated silencing of *hsp90* in *N. benthamiana* completely blocked I-2 triggered cell death thereby revealing the role of HSP90 in I-2 mediated defense response [27]. The role of HSP90 as a component of the defense response pathway was further demonstrated using BSMV-based VIGS, in the resistance gene *Mla13*-mediated resistance pathway in barley [46]. Silencing of *hsp90* in barley resulted in increase in hyphal growth, when challenged with the powdery mildew fungus *Blumeria graminis*, indicating that HSP90 is essential in the above signaling process. BSMV-based VIGS vector was also used to test the roles of the wheat genes *rar1*, *sgt1* and *hsp90* in downstream signal transduction of the R gene *Lr-21*-mediated resistance response in wheat against the fungal pathogen *Puccinia triticina* [97]. Silencing led to enhanced susceptibility towards the pathogen indicating that *M1a13*- and L*r21*-mediated pathways in barley (in which the role of the above genes are already demonstrated) and wheat share several common components.

VIGS has been used to reveal the role of several new components of defense pathways. These include NbGSTU1

(glutathione S-transferase) and NbACO1 (1-aminocyclopropane-1-carboxylic acid oxidase) from *N. benthamiana* active against the fungal pathogen *Colletotrichum orbiculare* [26,100], plastidic carbonic anhydrase against *P. infestans* [90], tomato calmodulin-like protein APR134 against *P. syringae* pv. tomato [20], and the EIL2 transcription factor and glutathione synthetase of *Nicotiana megalosiphon* against tobacco blue mold (*Peronospora hyoscyami f. sp. tabacina*) [13]. The role of tomato genes against the holoparasitic plant *Cuscuta reflexa* was revealed through silencing of *attAGP*, which resulted in significant reduction in its attachment capability to the plant [5]. Silencing of aconitase, an enzyme which catalyzes the reversible isomerization of citrate to isocitrate via the intermediate product cis-aconitate, led to delayed HR in *N. benthamiana* plants expressing AvrPto, following challenge with *P. syringae* pv. tabaci [76]. This suggested that aconitase plays a role in resistance-associated HR. *N. benthamiana* plants, in which a metacaspase, *NbMCA1* was silenced by VIGS showed increased susceptibility to *Colletotrichum destructivum* [43]. In barley and Arabidopsis, mutations in *mlo* genes, encoding a novel type of plant-specific integral membrane proteins, result in broad-spectrum powdery mildew resistance. Silencing of *Slmlo1* conferred enhanced powdery mildew resistance in wild-type tomato suggesting that the loss of function of this gene is responsible for susceptibility to powdery mildew, caused by *Oidium neolycopersici*, in tomato [9]. Silencing of Stearoyl acyl carrier protein desaturase-catalyzed synthesis of oleic acid in soybean resulted in constitutive expression of defense genes and showed resistance to bacterial and oomycete pathogens [56]. These results suggest that soybean probably responds to elevated levels of oleic acid by inducing a novel broad-spectrum resistance-conferring pathway. Silencing of a small peptide of a novel family of proteins in barley, BLUEFENSIN (BLN1), using the BSMV-based vector enhanced plant resistance to *B. graminis* sp. *hordei* indicating that BLN1 is a negative regulator of the basal defense response to powdery mildew in barley [74]. VIGS of *NTH201*, a novel class II KNOTTED1-like protein gene (cloned from *N. tabacum* cv. Xanthi) in *N. benthamiana* demonstrated that this protein plays a role in the cell-to-cell movement of TMV [114]. In a recent report, VIGS was used to confirm the identity of a cloned *R* gene *Rpp4* of soybean (*Glycine max*), responsible for mounting resistance against the fungus *Phakospora pachyrhizi*, the causative agent of the Asian soybean rust [75]. A BPMV-derived VIGS vector, carrying a portion of a cloned candidate gene (*Rpp4*) was inoculated on resistant lines of soybean plants carrying the *Rpp4* gene. Subsequent inoculation with spores of *P. pachyrhizi* led to development of infection only on the inoculated plants and not on the plants inoculated with the empty VIGS vector. This provided strong evidence that the insert carried in the VIGS vector was indeed part of *Rpp4*.

5.2. PCD related to plant development

Programmed cell death (PCD), an integral part of the life cycle in multicellular animals and plants, occurs not only during interactions with pathogen but also during developmental processes such as senescence, embryogenesis and development of vascular tissue. Evidence suggests that ubiquitin/proteasome pathway plays an important role in PCD. Silencing of 26S proteasome subunits, *NbPAF* and *NbRpn9*, in *N. benthamiana* led to the activation of cell death in plants [62] suggesting that controlled inhibition of proteasome activities may be involved in developmentally or environmentally-activated plant cell-death programs. Downregulation of *NbRpn9* displayed extra leaf vein formation with increased xylem and decreased phloem [54]. Given the fact that 26S proteasome is known to be player in PCD it is possible that RPN9 regulates plant vascular development by targeting a subset of regulatory proteins for degradation.

Hexokinase, an important enzyme in the basic carbohydrate metabolism, catalyzes phosphorylation of hexose, senses glucose levels and transmits sugar signals to the nucleus in plants. It has been reported recently that in animals, mitochondrial hexokinase plays major role in regulation of PCD [12,31]. Silencing of *Hxk1*, a mitochondrial hexokinase in *N. benthamiana* resulted in the formation of necrotic lesions on leaves, abnormal leaf development and reduced plant height [61]. Silencing was associated with the characteristic features of PCD including nuclear condensation and DNA fragmentation. The levels of ROS, pathogenesis-related proteins and other cell-death related genes, which are indicators of PCD, were higher in the *Hxk1*-silenced plants. These observations strongly suggested that *N. benthamiana Hxk1* plays an important role in the regulation of PCD in plants.

Prohibitins, a highly conserved class of mitochondrial proteins, are known to play important roles in cell cycle and senescence in animals and yeast. Silencing of prohibitin homolog, *PhPHB1*, in petunia led to late flowering, with dwarfed, curling leaves and displayed abnormal anther and pollen development [19]. The flowers of the prohibitin-silenced plants senesced earlier than control flowers and showed a higher rate of respiration throughout their life cycle. The higher respiration rates of the silenced flowers suggest that prohibitin may play a role in the normal functioning of mitochondria in plants as has been reported for animal systems. Thus, in plants, prohibitins seem to play important roles in cell cycling and aging as established for other systems. Silencing of another prohibitin homolog, NbPHB2, in *N. benthamiana* caused acute growth inhibition, leaf yellowing and symptoms of cell death [3]. The silenced plants were more susceptible to various oxidative stress-inducing agents. From these results it has been suggested that prohibitins play a crucial role in mitochondrial biogenesis, protection against stress and senescence in plants.

5.3. Plant growth and development

The roles of a number of genes related to plant growth and development have been revealed by the use of VIGS. For example, VIGS of the *N. benthamiana* gene DEFICIENS (*NbDEF*), an ortholog of Arabidopsis APETELA 3 and *Antirrhinum* DEFICIENS, genes playing important role in floral morphogenesis, led to defects in flower development [67], the mutant phenotype resembling that of Arabidopsis *ap3* and antirrhinum *def* loss-of-function mutants.

MADS-box genes represent a large family of transcription factors and are known to have critical role in several developmental processes in plants, including floral development. The TRV-LIC [30] discussed earlier has been used in *N. benthamiana* to investigate the role of *NbMADS4-1* and *-2* in flowering. Silencing of *NbMADS4-1* and *NbMADS4-2*, two MADS-box transcription factors, individually or simultaneously, resulted in loss of floral identity and determinancy and resulted in green, bushy flowers. This indicated that *NbMADS4-1* and *-2* have critical, non-redundant roles in floral development [30]. Silencing of *NbECR*, encoding for enoyl-CoA reductase enzymes catalyzing the last biosynthetic step for very long chain fatty acids (VLCFAs), in *N. benthamiana* produced necrotic lesions with typical cell-death symptoms in leaves [84]. The affected leaves had a disorganized cellular membrane structure indicating the essential role of very long chain fatty acids in membrane biogenesis.

Silencing of *NbNAP1*, the *N. benthamiana* homolog of *AtNAP1*, encoding for a SufB protein involved in Fe–S cluster assembly and expressing ubiquitously in plant tissue, resulted in yellowing of leaves and reduced numbers of chloroplasts [2]. However, silencing did not lead to any decrease in the expression of nuclear genes for chloroplast-targeted proteins and chlorophyll biosynthetic enzymes. In *N. benthamiana*, nuclear genes *NbERS* and

NbSRS encode for the organellar glutamyl tRNA synthetase and seryl tRNA synthetase respectively. Silencing of the genes led to severe yellowing of leaves and abnormal leaf morphology with a concomitant decrease in the transcript levels of the target genes. The numbers and sizes of the chloroplast and mitochondria were also reduced [60].

BTF3 is a sub-unit of nascent-polypeptide-associated complex that has been implicated in regulating protein localization during translation. Silencing of *NbBTF3* (-NAC) resulted in reduction in sizes of chloroplast and chlorophyll content in *N. benthamiana*. Transcription of several plastid and mitochondrial genes was reduced in *NbBFT3* silenced plants [113]. Silencing the gene *ispe*, whose product is involved in the plastidic methylerythritol phosphate (MEP) pathway-mediated biosynthesis of isoprenoid compounds led to developmental defects of chloroplast and mitochondrial biogenesis [4].

VIGS was used to demonstrate the role of Obtusifoliol-14a-demethylase (*CYP51*) in sterol biosynthesis in *N. benthamiana*. Silencing of the gene resulted in the accumulation of obtusifoliol, the substrate of *CYP51* with a concomitant growth reduction phenotype, indicating its function in normal plant growth [16]. Similar approaches, used for sterol 4α-methyl oxidase (SMO), of the sterol biosynthesis pathway, in which silencing of two genes *SMO1* and *SMO2* led to accumulation of distinct sterols in *N. benthamiana* suggested that in plants different families of SMOs are associated with the biosynthesis of distinct sterols [25].

The role of (E)-4-hydroxy-3-methylbut-2-enyl diphosphate synthase (HDS) and isopentenyl/dimethylallyl diphosphate synthase of the isoprenoid biosynthesis pathway encoded by *IspG* and *IspH* genes respectively has been validated in *N. benthamiana* by VIGS [83]. Silencing of both the genes resulted in albino leaves that had less than 4% of the chlorophyll and carotenoid pigments of control leaves and the leaves displayed a disorganized palisade mesophyll, reduced cuticle, fewer plastids, and disrupted thylakoid membranes.

The physiological role of P23k, a protein involved in sugar translocation and/or sugar metabolism in barley, was investigated by silencing by the BSMV-based vector [81]. This resulted in abnormal leaf development, asymmetric orientation of the main veins and cracked edges. Taken together with the fact that the distribution of P23k coincides with the distribution of cell wall polysaccharides, it has been proposed that P23k may be involved in the synthesis of cell wall polysaccharides in barley and contributes to secondary wall formation in barley leaves.

Silencing of the gene encoding Geranyl diphosphate synthase, participating in the isoprenoid biosynthetic pathway in tomato led to severely dwarfed plants and a decrease in the gibberellin content thus establishing a link between geranyl diposhphate and the gibberellin biosynthesis pathway [109]. The role of tomato abscission-related polygalacturonases was demonstrated by silencing, which resulted in delayed abscission and increased break strength of the abscission zone [52].

5.4. Abiotic stress

VIGS has been used to study the pathways responsible for abiotic stress response in plants. The ortholog of putative cDNA of late embryogenesis abundant gene *lea4*, known to be involved in stress tolerance in groundnut (*Arachis hypogea*) was silenced in tomato using VIGS [99]. Silenced plants showed enhanced susceptibility to moderate moisture stress as assessed by cell viability, superoxide radical activity and cell osmotic adjustments. In a related study differentially expressed cDNA clones from water-stressed peanut plants were used for silencing the corresponding genes in *N. benthamiana* and tomato [98]. Among the genes tested, silencing of flavonol 3-O-glucosyltransferase alcohol dehydrogenase, salt inducible protein and Heat shock protein 70 showed more visible wilting symptoms compared with the control plants during water deficit stress. Silencing of genes homologous to aspartic proteinase 2 and homolog of Jumonji class of transcription factor showed relative drought tolerant phenotypes. This study demonstrated the feasibility of using heterologous genes from VIGS-non-compliant plants, such as peanut for silencing in VIGS-compliant plants, such as tomato and *N. benthamiana*.

6. Limitations of VIGS

Although VIGS is a promising method of transient gene silencing, its very nature, i.e., the requirement to initiate viral infection, could restrict its application in some cases, especially in certain varieties of crop plants. For example, there are several viral resistance genes known in cultivated varieties of crops such as bean, cucumber, pea, pepper, potato, tomato, etc. [57], which confer resistance against certain viruses and thus, vectors derived from those may make VIGS ineffective. Moreover, appropriate biosafety precautions need to be practiced to prevent unintentional escape of the VIGS vectors into the environment if related viruses do not otherwise exist in the region/country where experiments would be conducted.

7. Conclusion

The VIGS technology has, to a great extent, fulfilled its promise of being a fast and efficient functional genomics tool. The increasing number of genes silenced in various plants using newly designed VIGS vectors is a testimony to its usefulness. Future progress will depend on the ability of investigators to extend the technique to more plant species and to develop high-throughput silencing methods in model plants and crops.

Appendix. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.plaphy.2009.09.001.

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